**Drosophila** mitochondrial transcription factor A (d-TFAM) is dispensable for the transcription of mitochondrial DNA in Kc167 cells

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We have cloned cDNA encoding *Drosophila* mitochondrial (mt) transcription factor A (d-TFAM). RNA interference (RNAi) of d-TFAM by lipofection of haemocyte-derived Kc167 cells with double-stranded RNA reduced d-TFAM to less than 5% of the normal level. Reflecting the ability of TFAM to stimulate mtDNA, RNAi of d-TFAM reduced mtDNA to 40%. Nonetheless, transcription of the ND2 and ND5 genes and their mRNAs remained unchanged for 8 days of the duration of RNAi. We thus show that d-TFAM is not essential for the transcription of *Drosophila* mtDNA.

Key words: HMG box, mitochondria, RNA interference.

INTRODUCTION

Human mitochondrial (mt) transcription factor A (h-TFAM; formerly referred to as h-mtTFA) is a factor of 29 kDa identified based on transcriptional activation of human mtDNA *in vitro*, and has two HMG boxes with a linker between them and a C-terminal tail sequence [1–3]. Many putative binding sites of h-TFAM have been demonstrated in the control region of human mtDNA, which contains heavy- (H-) and light- (L-) strand transcription start sites [15–17]. A nonanucleotide sequence. A factor has been suggested for the transcription of yeast the H-strand, but 3-fold for the light L-strand [9].

mtTFA was also cloned and found to stimulate the Xenopus transcription of 75 kb yeast mt genome [14]. These two proteins bind cooperatively to a nonanucleotide and another factor has been suggested for the transcription of yeast the H-strand, but 3-fold for the light L-strand [9].

Despite these lines of evidence for the essential role of TFAM, another factor has been suggested for the transcription of yeast and *Xenopus* mtDNA. In yeast, a core RNA polymerase of 145 kDa with sequence similarity to viral RNA polymerases of *Xenopus* transcripion of the ND2 and ND5 genes and their mRNAs remained unchanged for 8 days of the duration of RNAi. We thus show that d-TFAM is not essential for the transcription of *Drosophila* mtDNA.

**MATERIALS AND METHODS**

Screening and sequencing of *d-tfam* cDNA clones

The amino acid sequences of h-TFAM and x-TFAM were subjected to homology searching using the TBLASTN algorithm in the Berkeley *Drosophila* Genome Projects database. A fragment of cDNA encoding a sequence that was highly homologous with both h-TFAM and x-TFAM was found in the EST database. To isolate the full-length cDNA, a cDNA fragment was prepared by reverse transcriptase PCR of RNA extracted from Kc167 cells using paired primers, 5′-AGCTTACAACGCAAGGGC-3′ and 5′-GTGAAATTATGTGATGGAAAGAG-3′, based on the EST sequence. *A Drosophila melanogaster* cDNA library of 5 × 108 phage constructed from mRNAs from adult male and female flies (Stratagen) was screened in a buffer containing 50 mM Tris/HCl (pH 7.5), 5 × SSC (where 1 × SSC is 0.15 M NaCl/0.015 M sodium citrate), 5 × Denhardt’s solution, 1% SDS and 0.1 mg/ml salmon sperm DNA. After hybridization for 16 h at 65°C, filters were washed three times with 2 × SSC containing 0.1% SDS at room temperature and once with 0.2 × SSC.
containing 0.1% SDS at 65 °C for 30 min, and exposed to X-ray film (Fuji). cDNA was sequenced by the dideoxynucleotide method with an Applied Biosystems model 310 DNA sequencer.

Preparation of anti-d-TFAM antiserum
To express d-TFAM in Escherichia coli, a PCR fragment of d-tfam cDNA encoding the amino acid sequence from Glu-42 to Ile-257 was cloned into the BamHI-SalI sites of pGEX-2T (Amersham Pharmacia Biotech). Bacterial culture harbouring the plasmid was grown in LB medium containing 100 μg/ml ampicillin, and the expression was induced by 0.1 mM isopropyl β-d-thiogalactoside. The glutathione S-transferase fusion protein was purified using a glutathione-Sepharose 4B column (Amersham Pharmacia Biotech) according to the manufacturer’s instructions. New Zealand white rabbits were immunized by intramuscular injections of the purified protein (0.3 mg) in complete Freund’s adjuvant and boosted twice with the protein (0.3 mg) in incomplete Freund’s adjuvant. Serum was collected 6 weeks after the initial injection.

Cell culture and lipofection of dsRNA
Drosophila embryonic cell line Kc167 was maintained in HyQ CCM3 (HyClone Lab) serum-free medium at 25 °C. The lipofected cells were lysed in a buffer containing 10 mM Tris, 100 mM KCl, 100 mM NaCl, 1 mM EDTA, pH 7.4, and the expression was induced by 0.1 mM isopropyl-1-thiogalactoside. The glutathione S-transferase fusion protein was purified using a glutathione–Sepharose 4B column (Amersham Pharmacia Biotech) for 2 h. Total RNAs were extracted by TRIzol reagent (Gibco-BRL). RNA corresponding to 10 μg was separated in a 1.2% agarose/formaldehyde gel, blotted on to Hybond-N+ nylon membrane (Amersham Pharmacia Biotech) and hybridized to 32P-labelled Rp49, ND2 (nt 288–1045), ND5 (nt 6516–8053), mt rRNAs (nt 16S and 12 S rRNA; nt 12824–14756) or d-tfam (nt 119–892) at 42 °C in 5 × SSPE (standard saline phosphate/EDTA, 150 mM NaCl/10 mM sodium phosphate/1 mM EDTA, pH 7.4), 0.5% SDS, 5 × Denhardt’s solution, 50% formamide and 0.1 mg/ml denatured salmon sperm DNA for 16 h. The membrane was washed twice with 2 × SSC containing 0.1% SDS at room temperature and twice with 0.1 × SSC containing 0.1% SDS at 55 °C for 30 min, and then analysed by a Fuji Film BAS 2000 Image Analyzer. Genomic DNA was extracted by DNAzol reagent (Gibco-BRL). DNAs corresponding to 10 μg of extracted samples were digested with PstI and HindIII (Toyobo), electrophoresed in a 1% agarose/TAE (Tris/acetate/EDTA) gel, blotted to Hybond-N+ nylon membrane and hybridized to 32P-labelled ND5 (nt 6516–8053) or hml (DDBJ/EMBL/GenBank accession number AB035891; nt 6532–7260) at 65 °C in 50 mM Tris/HCl, pH 7.5/1% SDS/5 × SSC/5 × Denhardt’s solution/0.1 mg/ml of salmon sperm DNA for 16 h. The membrane was washed twice with 2 × SSC containing 0.1% SDS at 65 °C and twice with 0.2 × SSC containing 0.1% SDS at 65 °C for 30 min.

Preparation of dsRNA
The templates for the production of dsRNA were PCR-derived fragments sandwiched by double T7 promoter sequences. For the production of sense or antisense single-stranded RNA, PCR templates sandwiched by T3 and T7 sequences were transcribed using Klenow fragment of DNA polymerase I (New England Biolabs) and SP6 RNA polymerase (Stratagene), respectively. After treating with RNase-free DNase, RNA products were purified by phenol/chloroform extraction and ethanol precipitation. Although RNAs derived from double T7 promoter PCR templates self-annealed during synthesis, the annealing was completed by dissolving precipitated RNAs in annealing buffer (2 mM Tris/HCl, pH 7.5/2 mM EDTA), boiling for 2 min and keeping at room temperature for 16 h.

Western blotting
The lipofected cells were lysed in a buffer containing 10 mM Tris/HCl, pH 7.5, 2 mM EDTA and 1% SDS. Cell lysate corresponding to 10 μg of protein was separated by SDS/PAGE under reducing conditions using 12% polyacrylamide gel, and proteins were transferred to nitrocellulose membrane (Amersham Pharmacia Biotech). The membrane was blocked with PBS containing 5% skimmed milk for 1 h, reacted with 500-fold diluted antisera against d-TFAM for 16 h at 4 °C, washed extensively and reacted with 1000-fold diluted horseradish peroxidase-conjugated anti-rabbit IgG for 1 h. The ECL* Western-blotting system (Amersham Pharmacia Biotech) was used for the detection. Polyclonal antibody against Drosophila lamin B chain was obtained and used as described in [20].

Indirect fluorescence immunostaining of Kc167 cells
After labelling with MitoTracker Red CMXRos (Molecular Probes) according to the manufacturer’s instructions, Kc167 cells were fixed with methanol/water/acetic acid (95:4:1, by vol.) for 15 min. They were then permeabilized with cold methanol for 10 min, incubated with antisera against d-TFAM (1:500 in buffer A: PBS containing 0.1%, Tween 20) for 1 h, washed four times with buffer A, incubated with FITC-conjugated anti-rabbit IgG (Cappel; 1:500 in buffer A) for 1 h and washed four times with PBS. They were then mounted in 80% (v/v) glycerol containing 2 mM Tris/HCl (pH 8.0)/0.2 M 1,4-diazobicyclo[2.2.2]octane.

Northern and Southern blotting
Total RNA was extracted from the lipofected cells by TRizol reagent (Gibco-BRL). RNA corresponding to 10 μg was separated in a 1.2% agarose/formaldehyde gel, blotted on to Hybond-N+ nylon membrane (Amersham Pharmacia Biotech) and hybridized to 32P-labelled Rp49, ND2 (nt 288–1045), ND5 (nt 6516–8053), mt rRNAs (nt 16S and 12 S rRNA; nt 12824–14756) or d-tfam (nt 119–892) at 42 °C in 5 × SSPE (standard saline phosphate/EDTA, 150 mM NaCl/10 mM sodium phosphate/1 mM EDTA, pH 7.4), 0.5% SDS, 5 × Denhardt’s solution, 50% formamide and 0.1 mg/ml denatured salmon sperm DNA for 16 h. The membrane was washed twice with 2 × SSC containing 0.1% SDS at room temperature and twice with 0.1 × SSC containing 0.1% SDS at 55 °C for 30 min, and then analysed by a Fuji Film BAS 2000 Image Analyzer. Genomic DNA was extracted by DNAzol reagent (Gibco-BRL). DNAs corresponding to 10 μg of extracted samples were digested with PstI and HindIII (Toyobo), electrophoresed in a 1% agarose/TAE (Tris/acetate/EDTA) gel, blotted to Hybond-N+ nylon membrane and hybridized to 32P-labelled ND5 (nt 6516–8053) or hml (DDBJ/EMBL/GenBank accession number AB035891; nt 6532–7260) at 65 °C in 50 mM Tris/HCl, pH 7.5/1% SDS/5 × SSC/5 × Denhardt’s solution/0.1 mg/ml of salmon sperm DNA for 16 h. The membrane was washed twice with 2 × SSC containing 0.1% SDS at 65 °C and twice with 0.2 × SSC containing 0.1% SDS at 65 °C for 30 min.

Assay of in vivo transcription activity of mtDNA
After lipofection of dsRNA (4 days), Kc167 corresponding to 8 × 104 cells were rinsed twice with 10 mM Hepes buffer, pH 6.8, containing 140 mM NaCl and radiolabelled by incubating in 1.1 ml of the same buffer containing 1 mCi of [32P]P (Amersham Pharmacia Biotech) for 2 h. Total RNAs were extracted by TRizol reagent and the labelled RNA corresponding to 1 × 106 c.p.m. was hybridized to 2 μg of Bluescript vector, ND2, ND5 or mt rRNAs fragments which were pre-alkali treated, neutralized and dotted on to Hybond-N+ nylon membrane. The hybridization was carried out at 42 °C in a buffer containing 5 × SSPE, 0.5% SDS, 5 × Denhardt’s solution, 50% formamide and 0.1 mg/ml denatured salmon sperm DNA for 16 h. The membrane was washed twice with 2 × SSC containing 0.1% SDS at room temperature and twice with 0.1 × SSC containing 0.1% SDS at 55 °C for 30 min, and analysed by a Fuji Film BAS 2000 Image Analyzer.

RESULTS
Cloning of d-tfam
A BLAST search of the Berkeley Drosophila Genome Projects database yielded an EST fragment encoding an amino acid sequence homologous with x- and h-TFAMs. The full-length cDNA had an open reading frame encoding the 257-amino-acid sequence, shown aligned in Figure 1 with those of x- [9], h- [3] and m-TFAMs [21]. The PSORT II algorithm [22] predicts the...
Drosophila mitochondrial transcription factor A

Figure 1 Alignment of d-TFAM with other TFAMs

The N-terminal signal sequence, two HMG boxes, linker and C-terminal regions of the deduced amino acid sequence of d-TFAM are compared with those of TFAMs of other species. Conserved residues among the four species are printed on a black background. Other conserved residues are highlighted with a grey background.

Figure 2 Effect of RNAi on d-TFAM level in Kc167 cells

Kc167 cells were either not lipofected (cont) or lipofected with odds-paired (opa) or d-tfam (ds) dsRNA. Cell lysate (10 μg) was subjected 4 days later to SDS/PAGE using 12% (upper panel) or 4% (lower panel) polyacrylamide gel and blotted with rabbit serum antibody against d-TFAM or Drosophila lamin β-chain (lanβ), respectively.

N-terminal 41 residues to be a mt targeting signal, which is followed by HMG box 1 (Pro-48–Tyr-118), a linker region (Asp-119–Glu-152), HMG box 2 (Leu-153–Trp-222) and a C-terminal tail (Glu-223–Ile-257). Overall similarity of the sequence to the α-, h- and m-TFAM sequences was 45, 36 and 39%, respectively. The sequence predicts sizes of 29961 and 25748 kDa and pI...
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Figure 4 Effect of RNAi on the level of mtDNA

Total DNA (10 μg) of control Kc167 cells (cont) or Kc167 cells 4 days after lipofection with odd-s-paired (opa) or d-tfam (ds) dsRNA was digested with PstI and HindIII restriction enzymes, separated in agarose/TAE gel, blotted on to nylon membrane and hybridized with radiolabelled ND5 (mtDNA) or hml (control) probes.

values of 10.41 and 10.28 for the full-size and targeting-signal-clipped forms, respectively. Antiserum raised against a recombinant protein of Glu-41–Ile-257 detected, by Western blotting of Kc167 cell lysate, a weak band and strong doublet bands migrating within the range of 34–29 kDa (Figure 2, upper panel, control). Relatively slower migration in SDS/PAGE may be due to high content of basic amino acids. Whereas the weak band can be attributed to the precursor form before localization in mitochondria, the reason for detecting the doublet bands is unknown. Two alternatively spliced transcripts of m-tfam were found to produce m-TFAM and a testis-specific nuclear HMG-box protein lacking the mt targeting signal [21], but indirect fluorescence immunostaining of Kc167 cells with the antiserum showed signals that overlap considerably with dye that stains living mitochondria (MitoTracker; Figure 3).

Suppressed level of d-TFAM by lipofection of Kc167 cells with d-tfam dsRNA

Lipofection of Kc167 cells with d-tfam dsRNA specifically suppressed both of the doublet bands of d-TFAM. Figure 2 depicts Western blotting of the cell lysate 4 days after the lipofection. The signal for the doublet bands was decreased to one-twentieth whereas the signal for laminin β-chain, used as an internal control, remained unchanged. The effect was sequence-specific since lipofection of dsRNA encoding odd-s-paired (opa) showed no effect. Lipofection of single-stranded sense or antisense RNA encoding d-tfam showed no effect (results not shown). When the time course was followed by preparing cell lysate every other day, the suppression was evident 2 days after the lipofection and continued at least until the eighth day, when the effect started to be cancelled (results not shown).

Immunostaining of the cultures confirmed efficient RNAi of d-TFAM (Figure 3). Whereas most cells in control (Figure 3A) and odd-s-paired dsRNA-lipofected (Figure 3C) cultures were positive to d-TFAM staining, the signal disappeared from almost all cells in d-tfam dsRNA-lipofected culture (Figure 3E). Despite marked reduction of d-TFAM levels, however, the signal for living mitochondria remained unchanged (Figure 3F). This suggested that mt membrane potential was retained even under suppression of the d-TFAM level. Consistent with this observation, the cells lipofected with d-tfam dsRNA grew at a rate comparable with the control cells (results not shown).
that it took the Kc167 cells to double 10 times (8 days). The membrane potential across mt inner membrane was also not affected. Our results thus show that d-TFAM is not essential for transcription of Drosophila mtDNA. Since the properties of TFAMs may vary between species; however, we need to be careful in using the data to generalize about other TFAMs.

As a member of the HMG-box proteins, TFAM is implicated to stabilize mtDNA by wrapping around and condensing DNA [6,9,18]. Probably reflecting the same activity of d-TFAM, d-TFAM depletion in Kc167 cells resulted in reduced mtDNA levels. An evident phenotype of disrupting m-tfam in mice was mtDNA depletion, which was accompanied by respiratory chain deficiency [5]. It was possible that the mtDNA depletion was the direct consequence of m-tfam disruption and that the respiratory chain deficiency was an indirect consequence caused by reduction of the gene dosage.

Despite no apparent effect on mt mRNAs, d-TFAM depletion reduced the transcription of mt rRNA genes. To support the translation of all mt mRNAs, mt rRNAs are needed in large excess of mt mRNAs. Studies on HeLa cells indicated that the mechanism of H-strand transcription involves two overlapping, independently controlled transcription units starting at closely located sites, one covering the rRNA genes and the other covering the whole H-strand [23]. The differential activity of the two H-strand transcription units and an H-strand transcription attenuation event at the 3′-end of the 16 S rRNA gene account for the transcription of mt rRNAs at a rate 50 times higher than downstream mRNAs and tRNA genes [24]. Although nothing is known about the transcription of insect mt rRNAs, our result suggests that such a mechanism of mt rRNA transcription specified in insects is sensitive to gene dosage (mtDNA level).

**REFERENCES**


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**Figure 6 Effect of RNAi on the transcription activity of mtDNA**

Blue script vector (BS), ND2, ND5 and mt rRNA fragments (2 μg each) were dotted as duplicates on nylon membrane and hybridized with 1 × 10^6 c.p.m. of total RNA extracted from control cells or cells lipofected with d-tfam dsRNA (dsdTFAM). The photostimulated luminescence (PLS) count for each spot was calculated by a Fuji Film Image Gauge version 3.4.

**Reduction of mtDNA level while mt mRNAs levels were unchanged**

Figure 4 depicts the effect of d-TFAM depletion on the level of mtDNA. When normalized with the same amount of total cellular DNA, a Southern blot probed with the ND5 cDNA sequence showed a reduction of mtDNA to 40% in the cells 4 days after lipofection of dsRNA. This suggests destabilization of mtDNA in the absence of d-TFAM, probably due to its ability to wrap around distorted DNA. This level of 40% mtDNA was not transient because essentially the same result was obtained for the cells 6 days after lipofection (results not shown).

Consistent with no apparent change of phenotype in the mt membrane potential and the proliferation activity, the level of mt mRNAs remained unchanged in the d-TFAM-depleted cells, as monitored by Northern blot of ND2 and ND5 mRNAs (Figure 5). The result showed that d-TFAM is essential for the transcription of mtDNA. Slight reduction of 12 S and 16 S rRNAs might suggest a role for d-TFAM in the transcription of rRNA genes, but it is more likely due to the reduction of gene dosage.

Since it is well known that mt RNA is rather stable, the unchanged level of mt RNAs observed by Northern blotting could be simply due to their slow turnover. In order to address this question, newly synthesized transcripts were measured by pulse-labelling of the cells with [32P]p, and hybridization of radioactive RNA with DNA probes dotted on to nylon membrane (Figure 6). The radioactivity hybridized to ND2 and ND5 probes was virtually the same in control and dsRNA-lipofected cells. This result confirmed that d-TFAM is dispensable for the transcription of Drosophila mt mRNAs. On the other hand, the radioactivity hybridized to the mt RNA probe was decreased to half in the d-tfam dsRNA-lipofected cells.

**DISCUSSION**

Suppression of d-TFAM protein to less than 5% of the normal level by RNAi did not affect transcription of ND2 and ND5 genes and their mRNAs remained unchanged during the period
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